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Isolation and molecular characterisation of atypical enterotoxigenic *Bacillus cereus* with negative Voges-Proskauer reaction from Indian white shrimp *Fenneropenaeus indicus* (H. Milne Edwards, 1837)

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ABSTRACT

Enterotoxigenic *Bacillus cereus* is one of the important pathogenic organisms, which causes two distinct type of food poisoning in human beings *viz.,* emetic type and diarrhoeal type. As per biochemical tests, *B. cereus* is generally Voges-Proskauer (VP) positive. In the present study, two enterotoxigenic *B. cereus* isolates from white shrimp (*Fenneropenaeus indicus*) with negative VP reaction have been reported. Both the isolates were confirmed as *B. cereus* by other biochemical tests as well as molecular methods like PCR and 16S rRNA gene sequencing. They were also found to produce diarrhoeal enterotoxin by reverse passive latex agglutination (RPLA) test. The enterotoxin producing genes, *hbla* and *entb* were detected in both the isolates.

Keywords: *Bacillus cereus*, Enterotoxin, Voges-Proskauer (VP) negative

Introduction

Bacillus cereus group consists of five closely related species *viz., B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides* and *B. weihenstephanensis*. The organisms of this group are very closely related to each other and there is proposal to group them in a single species (Helgason *et al.*, 2000). *B. cereus* is a Gram positive spore forming organism, which causes two distinct type of food poisoning syndromes *viz.,* diarrhoeal type and emetic type, caused by two different types of toxins *viz.,* a heat-labile diarrhoeal toxin and a heat-resistant emetic toxin (Dierick *et al.*, 200s5). In some *B. cereus* outbreaks, there appears to be an overlap of diarrhoeal and emetic syndrome (Kramer and Gilbert, 1989). Apart from these, the involvement of *B. cereus* has also been reported to be associated with opthalmitis, respiratory tract infection and central nervous system disorder (Bekemeyer and Zimmerman, 1985; Beer *et al.*, 1990; Barrie *et al.*, 1992). Unlike other species of *Bacillus*, this pathogen can grow even under anaerobic condition. Food poisoning caused by enterotoxigenic *B. cereus* is very common in many European countries and accounts for 47, 33 and 22% of the total food poisoning cases reported in Iceland, Norway and Finland, respectively (Granum and Lund, 1997). Although, a major food poisoning outbreak due to this organism has not been reported in India, enterotoxigenic *B. cereus* has been isolated from variety of food items in the country (Kamat *et al.*, 1989; Agarwal *et al.*, 1997; Das *et al.*, 2009). This organism was also isolated from the stool specimen of 3.5% of the patients suffering from diarrhea in a study carried out in Kolkata, India (Banerjee *et al.*, 2011). *B. cereus* can produce biofilm, which is generally very resistant to various sanitisers (Oosthuizen *et al.*, 2002). Biofilm production on different food contact surfaces remains a constant source of contamination and a potential public health hazard. The conventional isolation and identification method of *B. cereus* involves isolation on selective agar containing polymixin B and egg yolk, followed by a battery of biochemical tests. As per biochemical tests, generally organisms under *Bacillus cereus* group are positive to Voges-Proskauer (VP) test (Rhodehamel and Harmon, 1998). In the present study, two atypical isolates of *B. cereus* have been reported with negative VP reaction.

Materials and methods

Isolation and identification of bacteria

Isolation of *Bacillus cereus* was done by plating on polymixin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA) (Szabo *et al.*, 1984). Identification of the isolates was carried out by performing motility test, catalase test, nitrate reduction test, lysozyme resistance, lecithinase reaction, mannitol fermentation test, glucose fermentation test *etc*. as mentioned in Bacteriological Analytical Manual (Rhodehamel and Harmon, 1998). Two of the isolates recovered from white shrimp (*Fenneropenaeus indicus)* procured from local market in Kochi were found to be negative for VP reaction and these

two VP negative atypical isolates were used in this study for further characterisation.

Screening for diarrhoeal enterotoxin production and haemolytic activity

Loopful of culture was inoculated into brain heart infusion broth and incubated overnight at 37°C with shaking. One milliliter of the culture was centrifuged at 5000 g at 4°C for 5 min and the supernatant was passed through 0.22µ membrane filter. The ability to produce diarrhoeal enterotoxin production was judged from the filtrate by reverse passive latex agglutination test using BCET-RPLA kit (Oxoid, U.K.) following manufacturer's instructions.

For checking β-haemolytic activity, loopful of culture was stab inoculated onto blood agar containing 5% sheep blood.

Screening for starch hydrolytic property

The starch hydrolysing property of both the isolates was screened by inoculation on starch agar to find a clear zone around the colony in case of positive reaction following exposure to the iodine vapour (Collins *et al.*, 2001).

Test for psychrotrophic growth

Both the strains were streaked on tryptic soya agar plate and incubated at 7°C for 7 days. The plates were observed daily for growth by visual examination up to 7 days.

Isolation of genomic DNA from the isolates

Isolation of genomic DNA was carried out by following the protocol of Mantynen and Lindstrom (1998) with slight modification. Briefly, 1.5 ml overnight grown culture in BHI broth with 1% (w/v) glucose was centrifuged at 5000 *g* for 10 min at 4°C, washed once with sterile normal saline solution and then suspended in 525µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and 45µl of freshly prepared lysozyme (50 mg ml^{-1}) was added, followed by incubation at 37°C for 30 min. Then 30µl of 10% SDS, 50µl of 10% N-laurylsarcosine (Sigma) and 5 µl of proteinase K (20 mg ml^{-1}) were added. The suspension was incubated at 37°C for 30 min, volume of the suspension was adjusted to 1 ml by addition of TE buffer, then 150µl of 5 M NaCl and 100µl of 10% CTAB solution in 0.7 M NaCl were added and heated at 65°C for 10 min. The suspension was extracted first with equal volume of chloroform: isoamyl alcohol (24:1) and then with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1). The nucleic acid was precipitated by addition of two volumes of chilled ethanol (-20°C). Then, it was kept at -20°C for 2 h and pelleted by centrifugation at 10000 *g* for 10 min at 4°C. The pellet was washed once with 70% ethanol, dried and then dissolved in 100µl of TE buffer. Then it was treated with 1µl RNase (10 mg ml^{-1}) and incubated at 37°C for 1 h and the DNA was stored at -20°C for future use.

The purity and concentration of purified DNA was determined by measuring OD at 260 and 280 nm in a UV spectrophotometer.

PCR assay for B. cereus group and different virulent genes

Both the isolates were subjected to PCR assays separately using *B. cereus* group specific primers as well as primers specific for three virulent genes *viz.*, *hbla*, *bceT* and *entFM*. The primers used in this study have been depicted in Table 1. One hundred nanogram of template was amplified in 25µl of reaction mixture consisting of 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTP, 1 U *Taq* DNA polymerase (Fermentas) and 10 pmol of respective primers. PCRs were performed using a thermocycler (Mastercycler, Eppendorf, Germany). In each PCR, the DNA isolated from *Staphylococcus aureus* ATCC 29213 was used as negative control. In each case, 30 cycles of amplification were carried out. The initial denaturation was at 95°C for 5 min and final extension was at 72°C for 5 min. The other details of cycling condition of each PCR are given in Table 1.

PCR amplification and sequencing of 16S rRNA gene for identification of species

The amplification of 16S rRNA gene was carried out using the primers 27F and 1525R (Rainley *et al.*, 1996). The sequences of 27F and 1525R are shown in Table 1. Approximately 200 ng of genomic DNA was amplified in 100 µl PCR reaction volume which contained IX PCR buffer (Fermentas), 2mM $MgCl_2$, 20 µM of each primer, 250 µM dNTPs and 4 U of *Taq* DNA polymerase (Fermentas). The PCR cycling condition consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles (details given in Table 1) and final extension was carried out at 72°C for 10 min.

The PCR product was detected in agarose gel electrophoresis after staining with ethidium bromide (0.3µg ml^{-1}). The PCR product was purified from agarose gel using QIAquick PCR purification kit (Qiagen, Germany) as per manufacturer's instructions. The purified PCR products were subjected to sequencing at SciGenom Lab Pvt. Ltd., Cochin, India.

Analysis of DNA sequence using BLAST

The deduced DNA sequences were compared with nucleotide resources of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) accessible at blast.ncbi.nlm.nih.gov.

Table 1. Details of primers used in the study

Primer	sequence	Target / Specificity and Reference	PCR cycling condition
BalF BalR	5'- TGCAACTGTATTAGCACAAGC T -3' 5'- TACCACGAAGTTTGTTCACTACT -3'	*Bacillus cereus* group (Chang *et al.,* 2003)	4°C for 45 seconds (denaturation), 55°C for 45 seconds (annealing) and 72°C for 45 seconds (extension).
HblA1 HblA2	5'- GCTAATGTAGTTTCACCTGTAGCAAC- 3' 5'- AATCATGCCACTGCGTGGACATATAA- 3'	*hbla* gene (Mantynen and Lindstrom, 1998)	94°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 min.
ENTA ENTB	5'- ATGAAAAAAGTAATTTGCAGG- 3' 5'- TTAGTATGCTTTTGTGTAACC- 3'	*entFM* gene (Asano *et al*., 1997)	94°C for 45 seconds, 52°C for 45 seconds and 72°C for 1 min.
BceT1 BceT2	5'- GAATTCCTAAACTTGCACCATCTC G- 3' 5'- CTGCGTAATCGTGAATGTAGTCAAT- 3'	*bceT* gene (Mantynen and Lindstrom, 1998)	94°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds
27 F 1525R	5'- GAGTM'GATCCTGGCTCAG - 3' 5'- AGAAAGGAGGTGATCCAGCC - 3'	For sequencing of 16S rRNA gene (Rainey *et al*., 1996)	94°C for 30 seconds; 52°C for 45 seconds and 72°C for 1 min 10 seconds.

BLAST comparison was done for both the sequences separately using megablast option of nucleotide BLAST (blastn).

Results and discussion

In biochemical tests, both the isolates were found to reduce nitrate, liquefy gelatin, motile, resistant to lysozyme, catalase positive and showed lecithinase reaction in media containing egg yolk (*i.e.*, PEMPA and PEMBA). Both the isolates utilised glucose anaerobically and possessed starch hydrolytic property as evident by the presence of clear zone around the colony following exposure to the iodine vapour.

The result of RPLA indicated that both the isolates were enterotoxigenic. Both of them were β-haemolytic on sheep blood agar, but none of them was found psychrotrophic as no growth was observed even after 7 days when incubated at 7°C.

In *B. cereus* group specific PCR, both the isolates yielded 533 bp amplified product. The *hbla* gene specific PCR using primers HBLA 1/HBLA2 yielded 834 bp product in both the isolates. The primer pair ENTA/ ENTB, which is specific for the *entFM* gene, showed 1.3 kb amplified product in both the isolates (Fig. 1). No amplified product was obtained in the *bceT* gene specific PCR using BceT1/BceT2 primers.

On PCR amplification of 16S rRNA gene using the universal primers 27F and 1525R, approximately 1.5 kb PCR product was obtained for both the isolates. The sequences of both the isolates (BC-shrimp-19 and BC-shrimp-39) are depicted in Fig. 2. The sequences have been submitted to NCBI and are accessible at

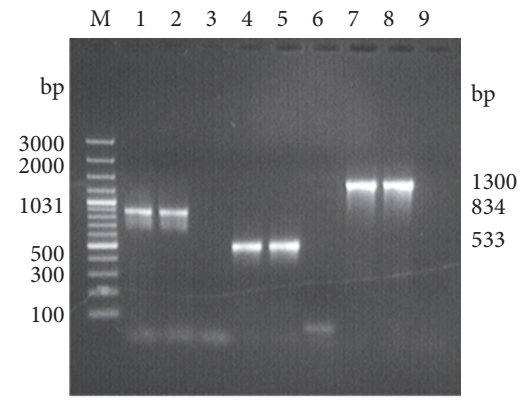

Fig. 1. *B. cereus* group and virulent gene specific PCRs

Lane M: 100 bp plus DNA ladder (Fermentas), Lane 1-3: *B. cereus* group specific PCR, Lane 4-6: *hbla* gene specific PCR, Lane 7-9: *entFM* gene specific PCR. Lane 1, 4 & 7: BC-19, Lane 2, 5 & 8: BC-39, Lane 3,6, & 9: *Staphylococcus aureus* ATCC 29213.

www.ncbi.nlm.nih.gov through the accession numbers JN676164 and JN676165 for BC-shrimp-19 and BC-shrimp-39, respectively.

On BLAST analysis, 16S rRNA gene of both the isolates showed almost 99% similarity with sequences of many *B. cereus* isolates. Apart from the organisms of *Bacillus* spp., the sequences of BC-shrimp-39 (JN676165) showed similarity with *Clostridium acetobutylicum*, while the sequences of BC-shrimp-19 showed similarity with *Enterobacter cloaceae* and *Clostridium acetobutylicum*.

Enterotoxigenic *B. cereus* causes serious food poisoning and hence the prompt identification of this organism is of utmost importance. Like most of the species of *Bacillus*, *B. cereus* is also a spore forming

ATGCAGTCGAGCGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC-
CGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAAC-
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG-
GAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACG-
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCT-
CAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC-
GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC-
CTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC-
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT-
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT-
CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAG-
GCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACC-
CGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAG

(a) BC-shrimp-19 (Accession No. JN676164)

CGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGA-
TAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCG-
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC-
CGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT-
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT-
CATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTG-
TAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGT-
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA-
GAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA-
GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT-
TATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTA-
CATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGG-
TAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGG

(b) BC-shrimp-39 (Accession No. JN676165)

Fig. 2. 16S rRNA genes of two atypical *Bacillus cereus* isolates from white shrimp

organism. The spores are generally resistant to heat and therefore the risk of *B. cereus* cannot be ruled out even in case of heat-treated foods. Very often, heat treatment often helps in germination of spores of *B. cereus* leading to potential public health danger. Moreover, some strains of *B. cereus* are psychrotrophic in nature and grow at low temperature (Meer *et al.*, 1991; Te Giffel *et al.*, 1997). The conventional methods of identification of this pathogenic organism is observation of typical colony with lecithinase reaction on *B. cereus* agar (*e.g.*, PEMPA or PEMBA), followed by a series of biochemical tests (Rhodehamel and Harmon, 1998).

The pathogen *B. cereus* is generally a VP positive organism. VP test is considered as one of the important tests for identification of this organism. But in this current study, two enterotoxigenic isolates of *B. cereus* with negative VP reaction have been reported. Both the isolates resembled *B. cereus* by biochemical tests, evidence of enterotoxin production, PCR and 16S rRNA gene sequence similarity. Similarity was also found with *Enterobacter cloaceae* and *Clostridium acetobutylicum*. But, *Enterobacter cloaceae* is Gram negative organism and *Clostridium acetobutylicum* is an anaerobic organism and thus, both of them can be ruled out. The results of the present study indicate that VP negative enterotoxigenic *B. cereus* is present in tropical aquatic system of Cochin and the criteria of VP negative cannot rule out the identification of *B. cereus*. Previously, VP negative *B. cereus* has also been reported from pasteurised milk and dairy products (Te Giffel *et al.*, 1997; Wong *et al.*, 1998). Te Giffel *et al.* (1997) observed that one out of 143 isolates of *B. cereus* from pasteurised milk in household refrigerator in the Netherlands was negative for VP reaction. However, this seems to be the first report of isolation of VP negative enterotoxigenic *B. cereus* from seafood. Therefore, it is essential to perform other biochemical tests, PCR assay and test for enterotoxin production (*e.g.*, RPLA) even for VP negative suspected *B. cereus* isolates from seafood.

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